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Industrial application of fungal laccase produced by solid state fermentation of agro pulse by products

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Abstract
Laccase is a copper containing polyphenol oxidase that acts on a wide range of substrate. A laccase producing fungi (*Trichoderma spp.*) was isolated and subjected to purification, characterization and dye decolorization study. Laccase activity was highest when operated at the following condition, Bengal gram used as a solid state substrate, 7 days incubation, 25 °C temperature, and pH-6 and inoculum size 2%. Guaiacol and sodium acetate buffer were used to assay laccase production. The enzyme was partially purified by ammonium sulphate precipitation and dialysis method. The purified enzyme was obtained with a specific activity of 89.959 IU/mg protein and a final yield of 70.26%. The optimal pH and temperature of the laccase was recorded to be 5 and 45 °C respectively. The values of kinetic parameters K_m and V_{max} for purified laccase were noted at 0.055 and 37.037 respectively, for guaiacol as substrate. The enzyme activity of crude enzyme and partially purified enzyme was observed to be 18.181 IU/ml and 44.710 IU/ml respectively. 63.866% of decolorization was observed at 48 h with Congo red and 19.009% with Rhodamine B by laccase enzyme produced by *Trichoderma spp.* is able to decolorize Congo red dye and Rhodamine B and can be used in controlling environmental pollution.

Keywords: Laccase, Trichoderma, Guaiacol, Dye decolorization, Partial purification

1. Introduction
Laccase is an enzyme that has potential ability of oxidation. It belongs to those enzymes, which have innate properties of reactive radical production, and its utilization in many fields has been ignored because of its unavailability in the commercial field. There are diverse sources of laccase producing organisms like bacteria, fungi and plants. Textile, pulp and paper industries discharge a huge quantity of waste in the environment, and the disposal of this waste is a big problem. To solve this problem, work has done to discover such an enzyme, which can detoxify these wastes and is not harmful to the environment. Laccase use oxygen and produce water as by product. They can degrade a range of compounds including phenolic and non-phenolic compounds. They also have ability to detoxify a range of environmental pollutants.
Laccase are typically found in plants and fungi. Plant laccase participate in the radical-based mechanisms of lignin polymer formation [13, 29] whereas in fungi laccase probably have more roles including morphogenesis, fungal plant-pathogen/host interaction and stress defense and lignin degradation. Although there are also some reports about laccase activity in bacteria Solid state fermentation (SSF) is environmental friendly as it resolves the problem of solid wastes disposal. It has been generally claimed that product yields are mostly higher in SSF when compared to submerged fermentation (SmF). Production of these biocatalysts using agro-biotech substrates under solid-state fermentation conditions provide several advantages in productivity, cost-effectiveness in labour, time and medium components in addition to environmental advantages like less effluent production, waste minimization, etc [20]. There are several reports describing use of agro-industrial residues for the production of laccase e.g. Banana skin by *Trametes pubescens* [19], Coconut flesh, groundnut shells and groundnut seeds by *T. hirsute* [4]. Most of these wastes contains lignin or/and cellulose and hemicellulose, which act as inducers of the ligninolytic activities. Moreover, most of them are rich in sugars, which make the whole process much more economical. All these make them very suitable as raw materials for the production of secondary metabolites of industrial significance by microorganisms.

2. Material and Methods

2.1 Place of work

The present study entitled "Industrial application of fungal laccase produced by Solid State Fermentation of agro pulse byproducts" was conducted in Post-Graduate Laboratory, Department of Industrial Microbiology, Sam Higginbottom University of Agriculture, Technology and Sciences, Allahabad India.

2.2 Collection of soil and its preparation

In systematic screening program for isolation of fungi (*Trichoderma*) soil samples were collected from the upper crust of the soil. Soil samples (approximately 5g) were collected using clean dry and sterile polythene bag along with sterile spatula. 1g of the soil sample was dissolved in 10ml of water to make soil suspensions.

2.3 Isolation of laccase producing microorganisms

The media was prepared and sterilized in autoclave for 15 minutes at 121 ºC. After sterilization, added streptomycin (0.025 g) as an antibacterial agent to prevent the any bacterial growth. 1 ml of soil sample was taken from soil suspension and serially diluted up to 10^{-5} in sterile distilled water. Different dilutions (10^{-5}) were poured in sterile petri plates followed by pouring of the media. The plates were incubated at 25 ºC for 5 to 7 days.

For obtaining the purified culture of *Trichoderma* the selective media used was Potato Dextrose Agar Media [12]. The media was autoclaved for 15 min at 121 ºC. It was then poured into sterile petri plates and left to solidify. The isolates were transferred on the respective plates and incubated for 3-5 days at 25 ºC. PDA plates was observed for growth and development of brown colored precipitate in tannic acid containing plates and reddish hallow zone in guaiacol containing plates.

2.4 Selection of potent strain and identification

The entire isolated organisms were inoculated on petri plates containing PDA medium with tannic acid and observed for development of brown colored precipitate and was assessed on daily basis. The organism showing faster growth was selected as potent strain.

2.5 Solid state fermentation experiment

Five grams of each husk was taken in 250 ml Erlenmeyer flasks separately and were moisturized with salt solution dissolved in 10 ml of distilled water to set the desired moisture level. Media was then sterilized at 121 ºC for 1 h to provide proper cooking of the substrate and to increase its susceptibility to microbial attack. Media was cooled after autoclaving to room temperature and inoculated with the inocula of potent strain grown on potato dextrose broth and culture was incubated in static conditions at 25 ºC in an incubator. The content was mixed thoroughly four times daily during the fermentation period by gently hitting the flask bottom on the palm of the hand.

2.6 Laccase Extraction

After the given period of incubation, the fermented substrate broth was extracted for laccase by mixing with 50 ml of chilled phosphate buffer (50 mM, pH 6.0). Extraction was performed with 50 ml of chilled buffer solution under rotary shaking (100 rpm) at 25 ºC for 1 h. The homogenate was filtered through nylon cloth (200 mesh size) and the filtrate was centrifuged at 8000 rpm at 4 ºC for 15 min. The clear supernatant was used for estimating the laccase activity.

2.7 Optimization of laccase production in solid-state fermentation conditions

To evaluate the relative efficiency of the selected agro pulse husks as solid substrate, a series of fermentation experiments were designed and conducted. The strategy adopted for optimization of various process parameters influencing laccase yield including consecutive evaluation of parameters. Initially one parameter was evaluated and it was then being incorporated at its optimized level in the subsequent optimization experiments. Optimization was done under at incubation time, temperature, different pH and inoculum size.

2.8 Dye decolorization

Two synthetic dyes, Congo red and Rhodamine B were studied for decolorization. The partially purified laccase was used to test its efficiency in decolorization of textile dyes. 1.0 mg of Congo red and 1.0 mg of Rhodamine B was dissolved in 100 ml distilled water and mixed properly. 5ml of each suspension was taken separately and added 0.5 ml of enzyme in each test tube. Assay was carried out by incubating the enzyme with dyes for 0 hrs, 24 hrs and 48 hrs at 37 °C. After the reactions the absorbance of the tested dyes was measured at 540nm.

2.9 Statistical analysis

The data obtained was subjected to statistical analysis such as single factor ANOVA and the data were interpreted and analyzed to calculate significant difference in the same way by using one-way classification and conclusion was drawn on the basis of analysis of variance technique (ANOVA) at 5% level of significance.

3. Results and Discussion

3.1 Isolation of fungal strain for laccase production

Soil samples were collected from the different areas of SHUATS campus, total of 6 isolates were recovered from different soil samples collect. 1g of the soil sample was dissolved in 10ml of water to make soil suspension followed by inoculation of soil suspension on petriplates containing Trichoderma specific medium for the isolation of *Trichoderma* species. The culture was purified by repeated transfer to agar plates and grown at 25 °C for 7 days. Colonies of *Trichoderma spp.* were selected as white to green in appearance on Trichoderma specific. medium plates.. These cultures were then transfer to potato dextrose agar plates and screened for laccase production. (Plates 4.1)

(A) **(B)** **(C)**

Plates 4.1: Crowded plate of *Trichoderma* on Trichoderma specific medium

3.2 Screening of laccase producing *Trichoderma spp.*

PDA plates were observed for growth and development of brown colored precipitate in tannic acid containing plates (Plates 4.4) and reddish hallow zone in guaiacol containing plates. (Plates 4.5)

Total six petri plates containing potato dextrose agar media were screened for laccase production but only one (Isolate 3) plate showed positive result with tannic acid and guaiacol containing plates [6, 34] in the previous studies which is comparable with the present findings.

Plates 4.2: Growth of *Trichoderma* with brown colored zone on potato dextrose agar media using tannic acid as substrate

3.3 Identification of fungal strain using Lacto phenol cotton blue staining

The strain was identified by Lacto phenol dye method and observed under microscope [6]. It showed similar result by which the culture was identified as the *Trichoderma* species. (Figure 4)

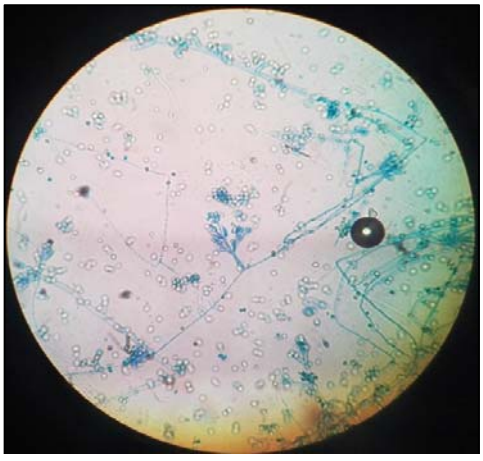

Fig 4.3: Lacto phenol cotton blue staining for identification of isolate 3 at 40x

3.4 Production of laccase from different Agro pulse byproduct

In the present investigation, optimization of the parameters for the production of laccase from the *Trichoderma spp.* under solid state fermentation using four commonly available agro-pulse husks *viz*. *Cajanus cajan* (red gram), *Vigna mungo* (black gram), *Phaseolus aureus* (green gram) and *Cicer artinum* (bengal gram) husks was evaluated as solid state substrate for the laccase production [23] are comparable to the present study in which *Cicer artinum* (bengal gram) was evaluated as potential solid state substrate for the maximum production of laccase.

Table 4.1: Production of laccase from different Agro pulse byproducts

S. No	Agro pulse used	Laccase Activity (IU/ml)
1	Red gram	35.92
2	Black gram	31.45
3	Green gram	26.96
4	Bengal gram	36.115

Due to substrate: F_{cal} (161.89) > F_{tab} (5.98) (S) at 5%

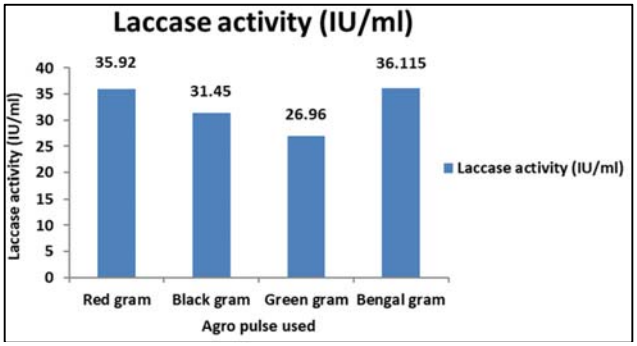

Fig 4.1: Production of laccase from different Agro pulse husk

3.5.1 Effect of incubation period on laccase production

The laccase activity was studied for the incubation period at 5 days, 7 days, 9 days and 11 days. Result of the investigation showed that laccase production increased with incubation time and further incubation showed reduction of laccase production (29.862 IU/ml) obtained at 7 days followed by 29.200 IU/ml at 9 days. The level of laccase increased gradually with increasing the incubation period up to a maximum at 7 days. It was observed that the maximum enzyme production from the fungus (*Trichoderma spp.*) was attained after 7 days (29.862 IU/ml) of incubation period.

Table 4.2: Effect of incubation period on laccase production

S. No	Incubation time (days)	Laccase activity (IU/ml)
1	5	26.225
2	**7**	**29.862**
3	9	29.200
4	11	22.837

Due to incubation time: $F_{cal}(0.403) < F_{tab}(9.276)$ (N.S.) at 5%

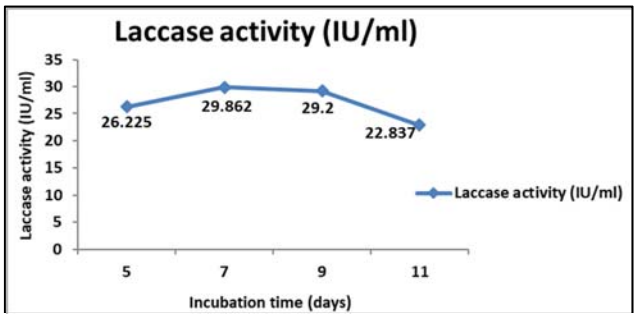

Fig 4.2: Effect of incubation period on laccase production

[10] Assayed that enhancement of laccase production from Trichoderma genus. After reported that the improvement of the physical parameters a time-course profile was carried out for 9 days. The samples were withdrawn at every 6 h intervals and laccase productions were determined. Laccase production increased gradually and achieved its maximum production of 12 U/ml after 9 days of cultivation. The enzyme production started decline thereafter [6]. examined that effect of different incubation periods on laccase productivity using Rice bran and Wheat bran under solid state fermentation conditions by Trichoderma genus was tested at time intervals of 2, 3, 4, 5, 6, 7 and 8 days. The level of laccase increased gradually with increasing the incubation period up to a maximum of 6 days. Then gradually decreased after these periods [15]. studied on Production of laccase enzyme for their potential application to decolorize fungal pigments on aging paper and parchment and observed that the maximum production of laccase enzyme by *Trichoderma harzianum* was observed after 6 days incubation and was nearly similar to the present work.

4.5.2 Effect of Temperature on laccase production

In the present investigation, optimization of temperature for the laccase production was studied at 25 °C, 30 °C, 35 °C and 40 °C. Effect of temperature were evaluated for the detection of optimum temperature required for the production of enzyme and the result showed optimum at 25 °C (29.45 IU/ml) and minimum was at 40 °C (22.56 IU/ml)**.**

Table 4.2.1: Effect of temperature on laccase production

S.No.	Temperature (°C)	Laccase Activity (IU/ml)
1	**25**	**29.45**
2	30	27.67
3	35	23.80
4	40	22.56

Due to Temperature: F_{cal} (0.120) <F_{tab} (9.276) (N.S) at 5%

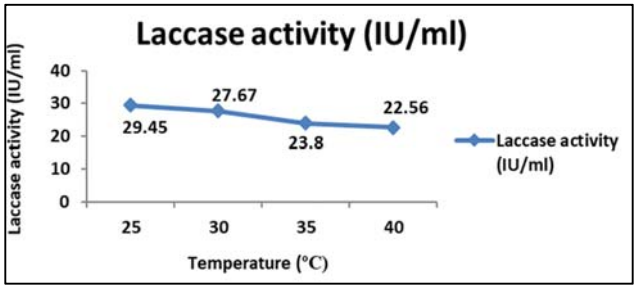

Fig 4.2.1 Effect of temperature on laccase production

Gnanasalomi and Gnanadoss (2019) also studied on laccase production and its optimization under solid state fermentation using cowpea pod as substrate and found the optimum temperature for laccase production at 30 °C [27]. worked on Effect of temperature and pH on growth of fungi Trichoderma harzianum and observed the *Trichoderma harzianum* was grown faster at 25-30 °C and it grow very slowly at above 35 °C and there is no growth at 45 °C. Optimum temperature of *Trichoderma harzianum* was found between 25 to 30 °C approx 28 °C by radial growth [15]. studied on Production of laccase enzyme for their potential application to decolorize fungal pigments on aging paper and parchment and observed that the maximum production of laccase enzyme by *Trichoderma harzianum* was at 35 °C after 6 days incubation.

4.5.3 Effect of pH on laccase production

In the present investigation, the optimum pH for growth and laccase production for most of the fungi has been reported to vary from 4.0 to 7.0. At pH 6.0 (36.287 IU/ml) recorded maximum enzymatic activity reported for laccase production from *Trichoderma spp*. which coincided with maximal cell growth and minimum laccase production was at pH 7.0 (30.523 IU/ml) (Table 4.6; Figure 4.5).

Table 4.2.2: Effect of pH on laccase production

S. No.	pH range	Laccase activity (IU/ml)
1	4	34.490
2	5	35.757
3	6	36.280
4	7	30.523

Due to pH: F_{cal} (0.384) < F_{tab} (9.276) (N.S.) at 5%

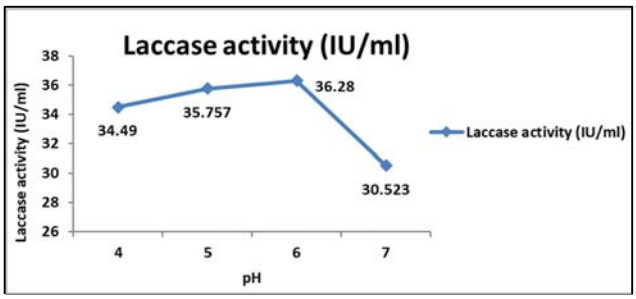

Fig: 4.2.2 Effect of pH on laccase production

[10] described isolation of laccase producing Trichoderma species and effect of pH and temperature on its activity, the laccase production by Trichoderma spp. was tested by

adjusting the pH of the PDA medium between 1.0-6.0; maximum laccase production (85 U/ml) was observed at pH 4.0 which coincided with maximal cell growth while minimum laccase production was observed at pH 6.0 (10 U/ml). Shifting the pH of PDA medium either to acidic or alkaline resulted in decreased production of laccase [15]. also studied on Production of laccase enzyme for their potential application to decolorize fungal pigments on aging paper and parchment and observed the effect of pH on laccase production by *Trichoderma harzianum* and found the maximum production of laccase enzyme by *Trichoderma harzianum* was observed at pH 5 after 6-day incubation.

4.5.4 Effect of inoculum size on laccase production

In the present investigation, the laccase activity was studied for the inoculum size from 2 ml to 5%. The enzyme was most active with 2% (30.082 IU/ml) inoculum size while least active with 5% (23.553 IU/ml) inoculum size.

Table 4.2.3: Effect of inoculum size on laccase production

S. No.	Inoculum size (%)	Laccase Activity (IU/ml)
1	**2**	**30.082**
2	3	26.660
3	4	23.746
4	5	23.553

Due to inoculum size: F_{cal} (0.191) < F_{tab} (9.276) (N.S.) at 5%

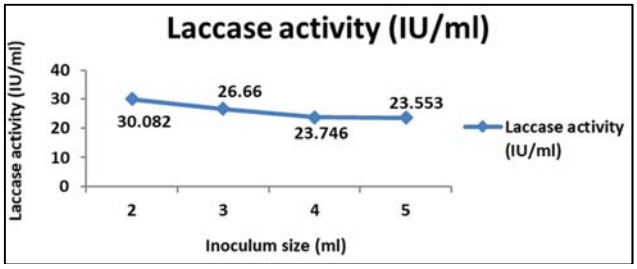

Fig: 4.2.3 Effect of inoculum size on laccase production

[31] studied on Optimization of culture parameters for hyper laccase production by *Trichoderma asperellum* by Taguchi design experiment using L-18 orthogonal array and observed the effect of inoculum size on laccase production and found that maximum laccase production was at inoculum size 5% using wheat bran as a substrate.

4.5.5 Production of laccase enzyme under optimized condition of different parameters

Summary of the various optimized fermentation conditions and laccase production is given in Table 4.3

Table 4.3 Optimum laccase production obtained under different fermentation conditions

S. No.	Fermentation conditions	Optimum laccase production (IU/ml)
1	Substrate (Bengal gram husk)	36.115
2	Fermentation period (7 days)	29.862
3	pH (6)	36.280
4	Temperature (25°C)	29.450
5	Inoculum size (2%)	30.082

4.6 Partial purification of laccase

The laccase present in the culture medium was purified by Ammonium sulphate followed by dialysis. The partial purified laccase was observed to be active over a wide range of pH 4 to 7. The activity of laccase present in crude culture medium produced by *Trichoderma spp.* was determined. The partially purified enzyme showed enzyme activity of 18.181 IU/ml in crude extract. After ammonium sulphate precipitation enzyme activity was 29.002 IU/ml. After dialysis showed the maximum enzyme activity 44.710 IU/ml. [35] reported that laccase from *Marasmius* species BBKA V79 was partially purified by ammonium sulphate precipitation. The purity of enzyme was 221.66-fold greater than the crude enzyme. Partial purification of laccase enzyme was performed by Ammonium sulphate precipitation followed by dialysis.

Table 4.4: Partial purification stages of laccase

S. No	Purification step	Laccase Enzyme activity (IU/ml)	Specific activity (IU/mg)	Protein purification fold	Enzyme yield (%)
1	Crude extract (28ml)	18.181	36.581	1	100
2	Ammonium sulphate ppt (14ml)	29.002	58.354	1.59	79.75
3	After Dialysis (8ml)	44.710	89.959	2.45	70.26

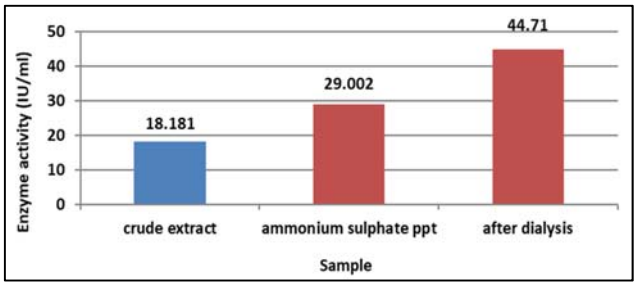

Fig: 4.3 Partial purification stages of laccase

4.7 Total protein estimation by Lowry's Method

Protein content in the sample was estimated by the Lowry's method**.** The total protein concentration is exhibited by a color change of the sample solution in proportion to protein concentration, which can be measured using calorimetric techniques. Optical density was measured at 530 nm using a UV-spectrophotometer. At last, absorbance of protein sample was measured and the concentration of the unknown sample was determined using the standard curve plotted.

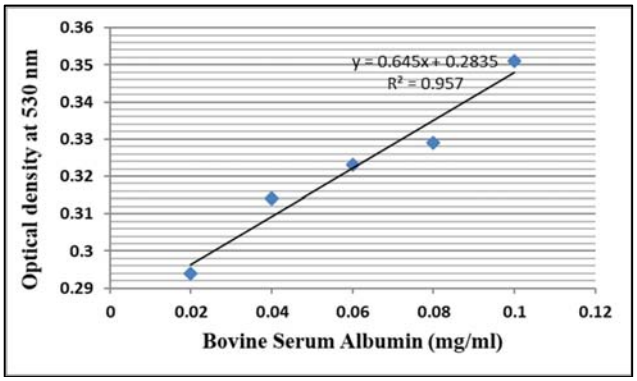

Fig: 4.4 Standard curve of Bovine Serum Albumin for protein estimation by Lowry's method

4.8 Characterization of Laccase enzyme

4.8.1 Thermal stability

The effect of temperature on laccase stability was determined by recording the absorbance of enzyme catalyzed reaction using guaiacol (2 mM) as substrate, dissolved in sodium acetate buffer (10 mM, pH 5.0), incubated at temperature 15, 25, 35, 45, 55 and 65 °C. The reaction mixture was incubated for 1 hr. The enzyme was found more stable at 45 °C after the completion of incubation.

Table 4.5: Thermal stability at different temperature

S. No	Temperature (°C)	Laccase activity (IU/ml) after 1 hr.
1	15 °C	30.891
2	25 °C	30.776
3	35 °C	31.123
4	45 °C	31.239
5	55 °C	30.776
6	65 °C	29.850

Due to thermal activity: F_{cal} (0.943) < F_{tab} (5.050) (N.S.) at 5%

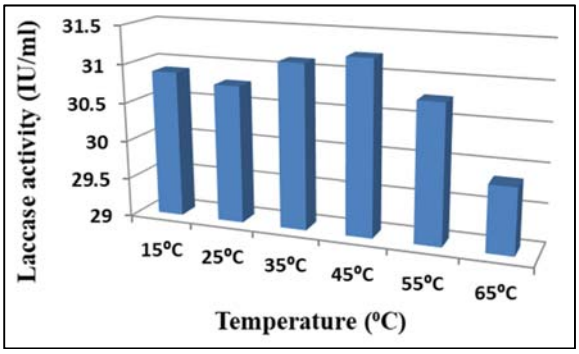

Fig: 4.5 Thermal stabilities at different temperature

[2] evaluate the effect of different temperature on ligninolytic enzymes (laccase and magnese peroxidase) in the range of 25 to 60 °C. For a variety of industrial applications, relatively high thermostability is an attractive and desirable characteristic of an enzyme. Temperature optimum for laccase and magnese peroxidase was observed at 50 °C and 30 °C respectively [6]. also studied the isolation of laccase producing fungi and partial characterization of laccase and obtained the optimal temperature for laccase from fungi was found to be 45 °C which is exactly similar to present investigation.

4.8.2 pH stability

The effect of pH on laccase stability was recorded. The absorbance of enzyme catalyzed reaction using guaiacol (2 mM) as substrate dissolved in buffers of different pH (0.1 M) buffers (sodium acetate buffer pH 4.0, 5.0; phosphate buffer pH 6.0, 7.0 and Tris HCl buffer pH 8.0). The incubation period for enzyme stability to study the effect of pH on laccase stability was 1 hr. at 4 °C and absorbance was recorded at 540 nm and residual activity was determined. The enzyme was found more stable at pH 5 (33.437 IU/ml).

Table 4.6: pH stability at different pH

S. No	pH	Laccase activity (IU/ml) after 1 hr.
1	4	32.511
2	5	33.437
3	6	32.743
4	7	32.511
5	8	31.586

Due to pH activity: F_{cal} (0.358) < F_{tab} (6.388) (N.S) at 5%

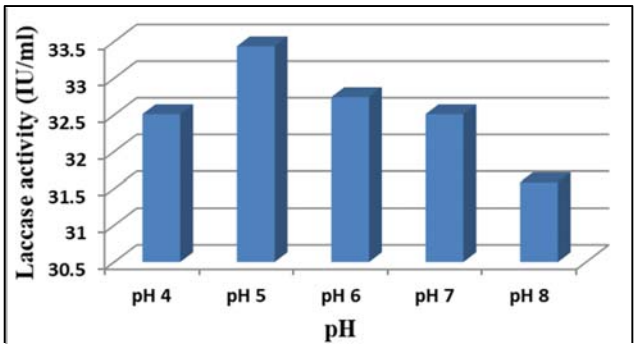

Fig: 4.5.1 pH stability at different pH

[18] also examined the effect of pH on enzyme activity and found the highest activity was observed at pH 4.5. Enzyme was able to hold back its activity in a long range of pH from 4.0 to 9.0. The purified enzyme exhibit maximum stability at pH range 5.0 to 8.5, comparable stability was also observed at pH 4.0, 4.5 and 9.0.

4.8.3 Kinetics constant of laccase enzyme

Kinetics constant of laccase enzyme was investigated using guaiacol (1 mM, 2 mM, 3 mM and 4 mM) as substrate, sodium acetate buffer (0.1 M, pH 4.0). Km and Vmax were calculated according to Lineweaver and Burk plot. The highest enzyme activity was found to be at 2 mM guaiacol concentration (35.983 IU/ml) while lowest enzyme activity was found at 1 mM guaiacol concentration (32.628 IU/ml).

The Km values of laccase from *Trichoderma sp.* towards the various concentration of substrate indicated that the binding affinities towards the different concentration of guaiacol were in the order: 2 mM > 3 mM > 4 mM > 1 mM.

The values of kinetic parameters Km and Vmax for partially purified laccase were noted at 0.055 mM and 37.037 µmol min^{-1} respectively for guaiacol as substrate with different concentration.

Table: 4.7 Kinetics constant of laccase enzyme

S. No	Guaiacol concentration	1/S	Laccase activity (IU/ml)	1/V
1	1 mM	1.0	32.628	0.03
2	2 mM	0.5	35.983	0.027
3	3 mM	0.33	34.363	0.029
4	4 mM	0.25	34.016	0.029

Due to guaiacol concentration: F_{cal}(1.623) <F_{tab}(9.276) (N.S.) at 5%

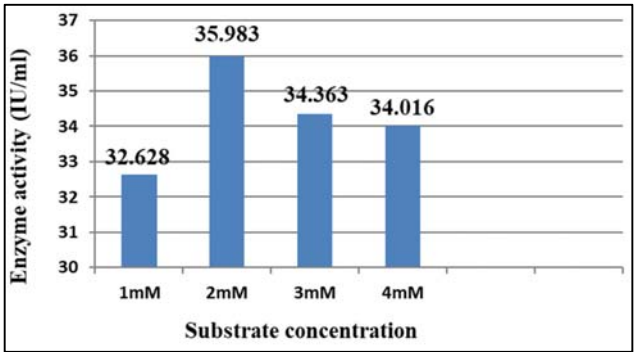

Fig: 4.6 Kinetics constant of laccase enzyme

[35] studied the kinetic properties of the laccase and find the value of Km and Vmax with the help of Line Weaver Burk plot and the kinetic parameters Km and Vmax of purified

laccase from *Marasmius sp.* BBKAV79 were found to be 3.03 mM and 5µmol min^{-1}, respectively.

4.8.4 Effect of metal ions on enzyme activity

To study the effect of various metal ions on enzyme activity, the enzyme was pre incubated for 15 min with 1 mM of each of the metal ions ($CuSO_4$, $MgCl_2$, $FeCl_2$, $MnSO_4$ and $CaCl_2$) prior to substrate addition, the remaining enzyme activity was done by standard enzyme assay protocol. The control was assayed without added metal ions. The highest enzyme activity was found with $CuSO_4.5H_2O$ (42.113 IU/ml) while lowest enzyme activity was found with $FeCl_2$ (35.172 IU/ml). The stability of enzyme activity against different metal ions was studied and it was observed that none of the metal ions stimulated the laccase activity. All the metal ions acted as the inhibitor of the enzyme in 1 mM concentration. Nearly 20.55% activity lost in the presence of Mg^{2+}, 21.07% with Mn^{2+}, 19.77% with Ca^{2+} and 21.33% with Fe^{2+} ion in 1mM concentration. The activity lost with Cu^{2+} ion was only 5.8% in 1mM concentration.

Table: 4.8: Effect of metal ions on laccase activity

S. No.	Metal ions	Enzyme activity (IU/ml)	Residual activity (%)	Activity lost (%)
1	Control	44.710	100	0
2	**$CuSO_4$**	**42.113**	94.191	5.8
3	$MgCl_2$	35.520	79.445	20.554
4	$FeCl_2$	35.172	78.666	21.333
5	$MnSO_4$	35.288	78.926	21.073
6	$CaCl_2$	35.867	80.221	19.778

Due to Metal ions: F_{cal} (1.000) < F_{tab} (6.388) (N.S.) at 5%

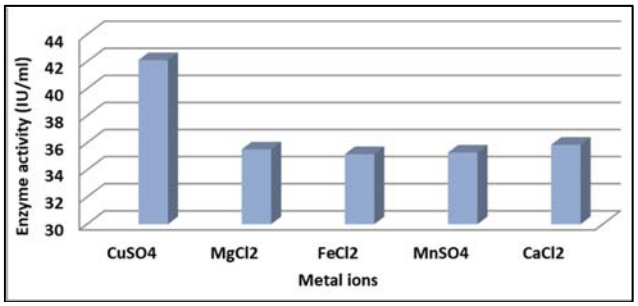

Fig: 4.7 Effect of metal ions on laccase activity

(18) also studied the purification and biochemical characterization of a newly produced yellow laccase from *Lentinus squarrosulus* MR13 and observed the effect of metal ions on enzyme activity and found in the presence of $CuSO_4$, the activity was increased ~ 5% and $CaCl_2$, $FeCl_2$, $MnSO_4$ act as inhibitor.

4.9 Dye decolorization

Two synthetic dyes, Congo red and Rhodamine B were studied for decolorization. The partially purified laccase was used to test its efficiency in decolorization of textile dyes. 1.0 mg of Congo red and 1.0 mg of Rhodamine B was dissolved in 100 ml distilled water and mixed properly. 5ml of each suspension was taken separately and added 0.5 ml of enzyme in each test tube. Assay was carried out by incubating the enzyme with dyes for 0 hrs, 24 hrs and 48 hrs at 37 °C. After the reactions the absorbance of the tested dyes was measured at 540 nm. The effect of dye decolorization was determined by the decrease in absorbance under the maximum wavelength of the dye. The efficiency of decolorization was expressed in terms of percentage.

$$\text{Decolorization (\%)} = \frac{\text{Initial absorbance - Observed absorbance} \times 100}{\text{Initial absorbance}}$$

In the present study, the decolorization of Congo red and Rhodamine B by laccase enzyme produced by *Trichoderma sp*. was studied at varying time interval from 0-48 h. from 0 h to 48 h, the maximum decolorization of congo red by laccase was observed at 48 h (63.866%) and the maximum decolorization of Rhodamine B by laccase was observed at 48 h (19.009%).

Table: 4.9 Percentage of decolorization by Laccase (%) with Congo red

S. No	Incubation time (hrs)	% decolorization
1	0	16.957
2	24	41.520
3	48	63.866

Due to decolorization by Congo red: F_{cal} (3185.044) > F_{tab} (19.000) (S) at 5%

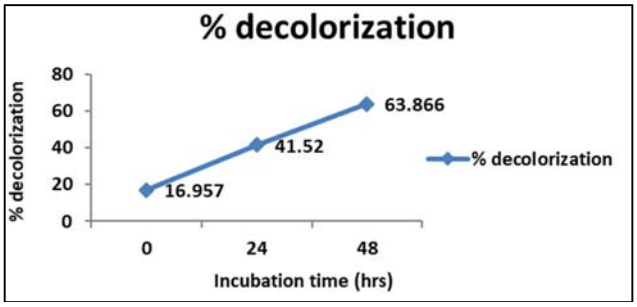

Fig: 4.8 Percentage of decolorization by Laccase (%) with Congo red

Table: 4.10 Percentage of decolorization by Laccase (%) with Rhodamine B

S. No	Incubation time (hrs)	% decolorization
1	0	3.270
2	24	11.341
3	48	19.009

Due to decolorization by Rhodamine B: F_{cal} (3.903) < F_{tab} (19.000) (NS) at 5%

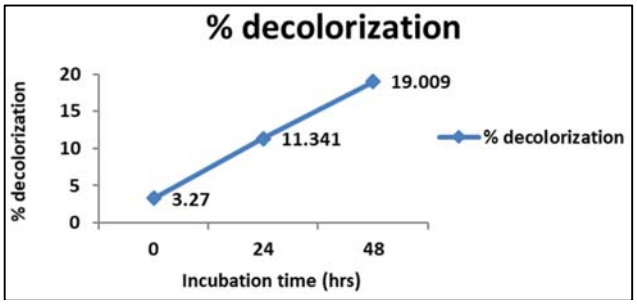

Fig: 4.9 Percentage of decolorization by Laccase (%) with Rhodamine B

[24] also studied the Congo red dye decolorization by partially purified laccase from *Pseudomonas aeruginosa* and observed the 42.86% of decolorization at 96 h by laccase enzyme.

Summary and Conclusion

- At different temperature, 25 °C showed maximum enzymatic activity (29.45 IU/ml) and on further increasing temperature enzymatic activity declined to 22.56 IU/ml at 40 °C. In case of 7 days incubation maximum enzymatic activity 29.862 IU/ml was shown and at 11 days incubation period enzymatic activity declined to 22.837 IU/ml.
- At pH 6 (36.523 IU/ml) maximum laccase production was observed while minimum production was at pH 7 (30.082 IU/ml). The laccase enzyme was most active with 2% inoculum size while least active with 5% (23.553 IU/ml) inoculum size.
- The enzyme was partially purified by ammonium sulphate precipitation and dialysis method. The laccase activity of crude extract was observed 18.181 IU/ml, after ammonium sulphate precipitation it was observed 29.002 IU/ml and after dialysis it was observed 44.710 IU/ml.
- The protein estimation was done by Lowry's method and protein estimated was 0.497 mg/ml.
- Specific activity was observed after dialysis 89.959 IU/mg and final yield was observed 70.26%. The protein purification fold noted after partial purification was 2.45 fold.
- The purified enzyme was most stable at temperature at 45 °C (31.239 IU/ml) while least stable at temperature 65 °C (29.850 IU/ml). The laccase enzyme was more stable at pH 5 (33.437 IU/ml) while least stable at pH 8 (31.586 IU/ml).
- Kinetic constant of laccase enzyme K_m and V_{max} was investigated using guaiacol as substrate, K_m and V_{max} was observed 0.055mM and 37.037 µM/min respectively.
- $CuSO_4.5H_2O$ showed maximum enzymatic activity 42.113 IU/ml followed by $CaCl_2$, $MgCl_2$, $MnSO_4$ and $FeCl_2$ which showed least enzymatic activity (35.172 IU/ml).
- 63.866% and 19.009% of dye decolorization was observed at 48 h with Congo red and Rhodamine B respectively by laccase enzyme produced by *Trichoderma spp* is able to decolorize Congo red and Rhodamine B and can be used in controlling environmental pollution.

From the above study it was concluded that, Bengal gram husk was the best laccase producing substrate among different agro byproduct under solid state fermentation using *Trichoderma spp*. Under different optimization conditions incubation period of 7 days was the best incubation period for laccase production, 25 °C was the best temperature, pH 6 was the best pH for laccase production and 2% inoculum size was the best inoculum size for laccase production. Partial purification was done by ammonium sulphate precipitation followed by dialysis. Protein was estimated by Lowry's method and found to be 0.497 mg/ml. Characterization of laccase enzyme was done under different temperature, different pH and metal ions. Kinetic constant was also determined and dye decolorization was done with two synthetic dyes Congo red and Rhodamine B which is beneficial for industry to minimize effluent and water usage and can be used in controlling environmental pollution.

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